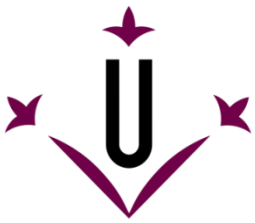

FUSARIUM MYCOTOXINS IN TOTAL MIXED RATIONS FOR DAIRY COWS

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ABSTRACT

Mycotoxins produced by certain fungal species of the *Fusarium* genus are frequently found as contaminants in cereals and feedstuffs. Fumonisins (FBs), deoxynivalenol (DON) and zearalenone (ZEN) are of special concern relative to animal health and productivity. The aim of this work was to analyse the levels of *Fusarium* mycotoxin contamination in samples of total mixed rations (TMRs) for dairy cows. To accomplish this analysis, an HPLC-MS/MS multi-mycotoxin method was developed and validated. The relation between the formulation of TMR samples and the presence of mycotoxins was also studied. From February 2016 to January 2018, a total of 193 TMR samples for dairy cows collected from farms located in different areas of Spain were analysed for the presence of FBs, ZEN, and DON and their metabolites. In total, 112 samples (58%) were contaminated with at least one mycotoxin, and 38 samples (20%) presented more than one mycotoxin. FBs were the mycotoxins most frequently found (34% positive samples). DON was detected in 17% of samples, and ZEN was detected in 16% of samples. Among the metabolites analysed, only deoxynivalenol-3-glucoside (DON-3-Glc) and 15-acetyldeoxynivalenol (15-ADON) were detected. The levels of all the *Fusarium* mycotoxins studied were always below the values recommended by the European Commission for feedstuffs. The wide variety of ingredients used in the formulation of the analysed samples made it difficult to reach definite conclusions, although it seemed that some cereal silages and concentrates such as cereals or compound feed used as ingredients of the TMR may be related to the presence of mycotoxins.

Keywords

Fusarium mycotoxins, total mixed ration, dairy cows, HPLC-MS/MS, multi-mycotoxin, feed ingredients

INTRODUCTION

Fusarium mycotoxins, which are usually synthesized before harvest, are frequently found as contaminants in cereals and feeds (Driehuis et al. 2008a). Among these *Fusarium* mycotoxins, trichothecenes, zearalenone (ZEN) and fumonisins (FBs) are of special concern regarding animal health and productivity (Streit et al. 2012). Ruminants are considered to be relatively resistant to *Fusarium* mycotoxins since these compounds are degraded by rumen microorganisms into less toxic metabolites (Pleadin et al. 2017). Moreover, the carry-over of these mycotoxins into milk has been reported to be low, although further research is needed (EFSA 2004a; EFSA 2005; Prelusky et al. 1984).

Deoxynivalenol (DON) is a type B trichothecene that is mainly produced by *F. culmorum* and *F. graminearum*. This mycotoxin inhibits protein synthesis and has cytotoxic and immunotoxic effects (Pestka 2007). Some studies have reported that this compound may affect the milk yield (EFSA 2017). ZEN is primarily produced by *F. graminearum* and is able to bind to oestrogen receptors as its chemical structure is similar to oestrogens, causing alterations in reproductive organs (CAST 2003). FBs are synthesized by *F. proliferatum* and *F. verticillioides* (Gallo et al. 2015), have hepatotoxic effects and can affect the production of milk (Fink-Gremmels 2008). The EU has recommended guidance values for DON, ZEN and FBs in products intended for animal feeding (EC 2006). Moreover, the European Food Safety Authority (EFSA) has published several opinions related to the presence of these mycotoxins in food and feed (EFSA 2004a, EFSA 2004b, EFSA 2005). Ruminants are likely to be exposed to a wide range of mycotoxins due to the heterogeneous composition of feeds. The total mixed ration (TMR) is a method of feeding dairy cattle based on the use of different known proportions of forages, commodities or by-products (for example, cottonseeds), grains, and protein supplements, minerals and vitamins. Some of these materials are frequently contaminated by different *Fusarium* species; thus, they are potential sources of DON, ZEN and FBs, among other mycotoxins. FB_1 and FB_2 are mainly present in maize (Tevell Åberg et al. 2013), and ZEN and DON are frequently found as contaminants in maize, wheat, triticale and oats (Döll and Dänicke 2011). DON can also be present in forages, especially in maize forage (Whitlow and Hagler Jr. 2005; Driehuis et al. 2008b; Ogunade et al. 2018). Consequently, mycotoxins usually co-occur in animal feed, resulting in toxic effects that could be antagonistic, additive or synergistic (Speijers and Speijers 2004).

Due to the complexity of feed matrices and the low level of contamination usually found in these samples, it is necessary to develop reliable analytical methods that enable the simultaneous detection of small amounts of different mycotoxins with high sensitivity. In recent years, high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) has become the most popular tool for multiple mycotoxin detection and quantification in food and feed due to its high specificity and selectivity (Zhou et al. 2016). As TMR is a mixture of different kinds of crops and materials, sample preparation and clean-up procedures are critical steps in the development of analytical methods, since it is necessary to minimize the matrix interferences as much as possible.

Several studies have been conducted to analyse the occurrence of mycotoxins in different feed ingredients, including in cereals and silages (Bottalico 1998; Placinta et al. 1999; Rasmussen et al. 2010; Pereira et al. 2014; Gallo et al. 2015; McElhinney et al. 2016; Pleadin et al. 2017; Panasiuk et al. 2019). However, very little research has been carried out on the incidence of mycotoxins in TMR intended for dairy cows (Pereyra et al. 2012; Yalçin et al. 2016; Cogan et al. 2017). The aim of the current study was to determine the frequency and levels of *Fusarium* toxins in TMR samples used as dairy cattle feedstuffs collected over two years. For this purpose, an easy and reliable method for the simultaneous detection of various mycotoxins by HPLC-MS/MS was developed and validated. The possible relation between the ingredients used for the formulation of TMR samples and the presence of the mycotoxins analysed was also assessed.

MATERIAL AND METHODS

Sampling

Samples of 193 different feedstuffs for dairy cows were collected from different areas of Spain (Cantabria, Castilla-León, Cataluña and Galicia) in the period from February 2016 to January 2018. TMRs were formulated by the farmers at each farm at the beginning of the day using a "unifeed system". Each farm provided a bulk sample of 4-5 kg of TMR generated by pooling elementary samples of 100 g that were taken from the daily batch as it was delivered by specialized technicians, as a representative fraction of the whole. Farmers were also asked to provide a report that contained the composition of the TMR, as well as the constituents of the compound feed used as one of the ingredients of the TMR. Out of 193 TMR samples received, a total of 163 included a report with the specific composition of the sample. The samples were stored at -18 °C until analysis.

92 **Chemicals, reagents and materials**

93 Acetonitrile and methanol, both HPLC grade, and *n*-hexane were obtained from Scharlab (Sentmenat,
94 Spain). Glacial acetic acid and ammonium formate were sourced from Fischer Scientific (Loughborough,
95 UK). Water was purified on a Milli-Q system (Millipore, Billerica, MA, USA). Standard solutions of
96 DON, 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), deoxynivalenol-3-
97 glucoside (DON-3-Glc), ^{13}C-labelled DON ($^{13}C_{15}$-DON), FB_1, FB_2, ^{13}C-labelled FB_1 ($^{13}C_{34}$-FB_1), ZEN, α-
98 ZEN, β-ZEN, and ^{13}C-labelled ZEN ($^{13}C_{18}$-ZEN) were supplied by Biopure (Coring System Diagnostix,
99 Gernsheim, Germany). Glass microfibre filters (Whatman No. 113) were purchased from Whatman
100 (Maidstone, UK). Multisep® 226 Aflazon+ Multifunctional columns were purchased from Romer Labs
101 Diagnostic GmbH (Tulln, Austria).

102 Mycotoxin stock solutions were made by diluting the standard solutions in methanol for FB_1, FB_2, DON,
103 ZEN, α-ZEN and β-ZEN and in acetonitrile for 15-ADON, 3-ADON, DON-3-Glc, $^{13}C_{15}$-DON, $^{13}C_{18}$-
104 ZEN and $^{13}C_{34}$-FB_1. The concentrations in the stock solutions were checked by UV spectroscopy
105 according to AOAC official methods of analysis (Horwitz and Latimer 2006). Working solutions of each
106 family of mycotoxins and one of the labelled mycotoxins at different concentrations were prepared using
107 the appropriate solvent. All solutions were stored at -4 °C.

108 **Sample preparation**

109 Feed samples were dried over 24 hours at 60 °C, ground into fine powder and stored at -18 °C until
110 analysis. An in-house validated method based on methods by Monbaliu et al. (2010) and Tevell Åberg et
111 al. (2013) was used. Two grams of feed sample were weighted into 50-mL polypropylene centrifuge
112 tubes, spiked with 250 µg/kg of $^{13}C_{15}$-DON (used as the internal standard) and allowed to stand for 30
113 min. Then, 30 mL of extraction solution consisting of acetonitrile:water:acetic acid (79:20:1, v/v/v) was
114 added. The samples were horizontally shaken for 1 hour at 200 rpm and centrifuged for 10 min at 1008 *g*.
115 Then, 15 mL of the supernatant were defatted by extraction with 10 mL of *n*-hexane for 10 min on a
116 rotatory shaker. Finally, after 10 min of centrifugation at 1008 *g*, the hexane layer was removed and
117 discarded.

118 To extract all the mycotoxins, two different clean-up pathways were performed. On the one hand, to
119 recover DON, ZEN, and their metabolites, 7 mL of the defatted extract was passed through a Multisep®
120 226 Aflazon+ Multifunctional column, and 3 mL of the clean extract was collected in a vial. On the other

hand, with respect to FB_1 and FB_2, 6 mL of the defatted extract was filtered through a glass microfibre filter, and 2 mL of the filtrate was combined with the Multisep® 226 eluate. The mixture was evaporated to dryness by nitrogen gas at 40 °C, and the residue was reconstituted in 1 mL of the mobile phase consisting of methanol:5 mM ammonium formate in water (50:50, v/v). Before the injection into the chromatographic system, the final extract was filtered through a 0.22-µm PTFE disposable syringe filter (Kinesis, Cambridgeshire, UK).

HPLC-MS/MS analysis

HPLC-MS/MS analysis was performed with an Agilent series 1290 RRLC system (Agilent, Santa Clara, CA, USA) equipped with a binary pump (G4220A) and an autosampler thermostat (G1330B) coupled to an Agilent triple quadrupole mass spectrometer (6460A) with an electrospray ionization (ESI) source (G1958-65138). Chromatographic separation was achieved using a Zorbax Plus C18 (1.8 µm x 2.1 x 100 mm) column from Agilent (San Jose, CA, USA). The MassHunter software (Agilent, Santa Clara, CA, USA) was used for optimization and quantification. Gradient elution was established with a mobile phase consisting of methanol (eluent A) and 5 mM ammonium formate in water (eluent B) at a flow rate of 0.2 mL/min. The gradient elution started with a linear increase of A, from 25% to 100% during 3.75 min, maintaining these conditions up to 6.0 min. The composition of the mobile phase returned to the initial conditions in 0.5 min and was maintained during 1 min, resulting in a total run time of 7.5 min. The temperature of the column was set at 25 °C, and the injection volume was 5 µL.

MS analyses were carried out using selected reaction monitoring (SRM) mode with positive and negative electrospray ionization ($ESI^{+/-}$). The source gas temperature and sheath gas temperature were set at 325 °C and 400 °C, respectively, with flows of 5 L/min and 11 L/min, respectively. The nebulizer was set to 45 psi. The capillary and nozzle voltage were 3500 V and 500 V, respectively. The retention time and MS/MS parameters are shown in Table 1.

Method development

The analytical method used was assessed for precision, calculating the apparent recovery, which is the ratio between the measured concentration and the spiked concentration. For this purpose, eleven blank feed samples were spiked with known amounts of mycotoxin mixtures at three different concentration levels (low, medium and high) at least in triplicate. Moreover, $^{13}C_{15}$-DON, $^{13}C_{34}$-FB_1 and $^{13}C_{18}$-ZEN were added to every sample in the same concentration (250 µg/kg) to be used as internal standards. As each

TMR sample had a unique composition, it was not possible to validate the method for every feed sample. Accordingly, a TMR sample, with an average composition, was considered a blank sample to calculate the apparent recovery of the method.

The matrix effect was studied by comparing the slopes when matrix-matched calibration solutions and solvent calibration solutions were prepared at concentrations between 0.5 and 250 µg/L. The matrix effect was calculated using the following equation: ME (%) = [(slope matrix/slope solvent) -1] x 100. For the estimation of the LOD and LOQ, extracted blank samples were spiked at low concentrations (from 0.1 to 50 µg/L). Both LOD and LOQ were calculated as the concentrations for which signal-to-noise (S/N) ratios were 3 and 10, respectively. The method performance for DON, DON-3-Glc, 3-ADON, 15-ADON, ZEN, α-ZEN, β-ZEN, FB_1 and FB_2 is summarized in Table 2.

Statistical analysis

Data processing was performed using JMP Pro 13.1.0. Analysis of variance (ANOVA, significance level set at $p < 0.05$) and multivariate partial least square (PLS) regression were applied to the data. In addition, the correlation among the concentration of mycotoxins under study and the components of the TMR samples was studied. Other calculations were performed using Microsoft Office Excel 2016.

RESULTS AND DISCUSSION

Development of the sample preparation method

The complexity of some animal feed matrices, coupled with the chemical diversity of the analytes, makes sample preparation challenging for the development of a multi-mycotoxin analytical method.

Based on the literature, different extraction procedures were evaluated in this work. Relative to the extraction solvent, various combinations of organic solvents, acetonitrile or methanol, and water were used. In agreement with previous studies, better results were obtained using acetonitrile than with methanol (Garon et al. 2006; Sulyok et al. 2006; Zhao et al. 2015). Moreover, it has been proved that the acidification of the extraction solvent improves the recovery of FBs (Bennett and Klich 2003; Sulyok et al. 2006) and prevents the degradation of certain analytes when acetonitrile is used (Mol et al. 2008). For these reasons, the extraction solvent was acidified with acetic acid. For the extraction method, different strategies were studied. It was not possible to develop a method without a clean-up step, owing to the matrix interferences. A defatting step was necessary to obtain sufficiently clean extracts. QuEChERS was

178 the first approach because it is a relatively fast, cheap and easy method, and because it has been
179 frequently used for mycotoxin analysis (Berthiller et al. 2018). For the clean-up, C18, $MgSO_4$ and
180 activated charcoal were tested as sorbents. ZEN could not be recovered in that way, and low recoveries
181 were obtained for the rest of the mycotoxins. Finally, the chosen method was based on the methods
182 developed by Monbaliu et al. (2010) and Tevell Åberg et al. (2013). A multifunctional column,
183 Multisep®226 Aflazon+, was used for the clean-up, since these columns had been proven to work well for
184 DON and ZEN in grains and animal feed (Tevell Åberg et al. 2013). Moreover, a second pathway using a
185 glass microfibre filter was necessary to recover FBs (Monbaliu et al. 2010). Therefore, a suitable multi-
186 mycotoxin method that allowed the analysis of TMR samples with only one LC-MS/MS injection was
187 developed (Table 2).

188 **Analysis of TMR samples**

189 The results of the analysis for the presence of mycotoxins in TMR samples are summarized in Table 3. In
190 total, 112 TMR samples (58%) out of 193 collected from February 2016 to January 2018 presented
191 detectable levels of some of the mycotoxins under study, and 38 (20%) were contaminated by more than
192 one mycotoxin. DON was found in 17% of the samples, ZEN in 16%, and FBs, the most frequently
193 detected toxins, in 34%. The presence of metabolites of DON (DON-3-Glc, 15-ADON, 3-ADON) and of
194 ZEN (α-ZEN, β-ZEN) was observed. Only DON-3-Glc and 15-ADON were detected in 2% and 9% of the
195 samples analysed, respectively. In none of these cases did the concentration of the toxins exceed the
196 guidance values recommended by the European Commission (EC 2006).

197 The statistical analysis (ANOVA) showed that there was a significant difference in the concentration of
198 ZEN ($p = 0.04$) and FBs ($p = 0.01$) in samples based on the geographic area where the samples had been
199 collected. Conversely, this difference was not significant in the case of DON. ZEN was not detected in
200 any of the samples analysed that were taken from farms located in Galicia (area 08). Regarding FBs, these
201 toxins were the most frequently detected, especially in samples received from Cantabria (area 04) and
202 Cataluña (area 25) (Table 4). In addition, two specific farms from these two areas presented an especially
203 high incidence of FBs in their samples, with 92 and 100% of the samples positive for the presence of FBs
204 and with concentrations ranging from 45 to 1100 μg/kg, and from 500 to 1000 μg/kg, respectively.
205 Looking at the composition of these specific TMR samples, we observed that those taken from the farm
206 in the area 04 contained a high percentage of maize as an ingredient of the compound feed, which also
207 represented a high portion of the total composition (25.6-33.9%). For the samples taken from the farm of

208 the area 25, all of them contained maize at a relatively high percentage (13.1-17.1%) compared to the
209 total set of samples. Maize silage, bagasse and barley were always present as ingredients of these
210 samples. FBs are frequently found contaminating maize (Marin et al. 2013; Tevell Åberg et al. 2013), so a
211 greater use of maize in these samples may be linked with the presence of FBs.

212 In some of the studies conducted involving a wide variety of feedstuffs, FBs were also the most
213 frequently detected mycotoxins. Pleadin et al. (2017) analysed the presence of *Fusarium* mycotoxins in
214 maize silage and concentrated feeds. DON was detected in 77%, ZEN in 66% and FBs in 80%. In
215 contrast, DON appeared to be the most common mycotoxin for Kocasari et al. (2012); 45% of samples of
216 dairy cattle feed contained DON, 35% ZEN and 10% FBs. In a similar manner, Driehuis et al. (2008a)
217 analysed different materials used as feedstuffs for dairy cattle. DON was the most frequently detected
218 mycotoxin, and the incidence was particularly high in compound feed and silage samples (53 and 54%
219 positive samples, respectively). ZEN was detected in 28% of compound feed samples and 17% of silage,
220 and FBs were detected in only 2 samples, one of compound feed and one of corn gluten feed sample.

221 DON and ZEN metabolites could also be detected with the analytical method developed. α-ZEN and β-
222 ZEN were not found in any of the TMR samples. DON derivatives, as the conjugated compound DON-3-
223 Glc and the acetylated forms 3-ADON and 15-ADON, have been reported to co-occur with DON in
224 cereal commodities (Berthiller et al. 2013). In the current study, 18 samples contained 15-ADON (mean
225 28 µg/kg). However, 3-ADON was not detected in any of the TMR samples. As far as DON-3-Glc was
226 concerned, only three samples presented detectable levels of this compound (mean 46 µg/kg). Moreover,
227 only one positive sample for DON presented DON-3-Glc, and 15-ADON co-occurred with DON in only
228 three samples. In contrast to our results, Monbaliu et al. (2010) reported a higher incidence of 3-ADON
229 and 15-ADON, pointing out that these metabolites did not always co-occur. Abdallah et al. (2017) found
230 DON-3-Glc in 10% of the feed samples, and Driehuis et al. (2008a) did not find any sample containing 3-
231 ADON or 15-ADON. Streit et al. (2013) found that DON-3-Glc always co-occurred with DON, and 75%
232 of the samples presented this toxin. DON-3-Glc level represented an average 12% of the DON
233 concentration, in agreement with the results obtained by De Boevre et al. (2012), who reported that DON-
234 3-Glc represented up to 14%. For Zachariasova et al. (2014), DON-3-Glc represented levels up to 7-20%
235 of the original DON concentration**.** In our research, the mean concentrations of DON-3-Glc and 15-
236 ADON were 17 and 10%, respectively, of the mean level of the concentration of DON in the positive
237 samples. The same *Fusarium* strains that produce DON could produce 3-ADON and 15-ADON, although

238 the pattern of production differs among strains, and their distribution also depends on the location and the
239 season of sampling (Payros et al. 2016). Moreover, the fact that none of the samples presented 3-ADON
240 could be affected by the lower percentage of recovery of the method employed (Table 2).

241 The co-occurrence of DON and ZEN in several crops and commodities has been widely reported
242 (Driehuis et al. 2008a; Njobeh et al. 2012; Zavhariasova et al. 2014; Abdallah et al. 2017). Both
243 mycotoxins are produced by *F. graminearum* and *F. culmorum*, and the requirements to produce them are
244 quite similar (Njobeh et al. 2012). Nevertheless, our outcomes showed that only four samples contained
245 DON and ZEN at the same time, and any sample that presented DON, ZEN and FBs simultaneously was
246 never found.

247 **TMR composition in relation to the mycotoxin concentration**

248 Feeds for dairy cows are formulated with a wide variety of ingredients, with forages and concentrates
249 being the main components of the ruminant diets. These materials could be potential sources of
250 mycotoxins. Therefore, it is an important issue to identify which ingredients used in the formulation of
251 the TMR samples contribute to the total mycotoxin load.

252 For this purpose, PLS regression and correlation analysis were carried out. PLS is a multiple regression
253 method that produces a model for each mycotoxin by combining all TMR components at a time, while
254 correlation analysis was carried out with one single variable at a time. Thus, the results of both analyses
255 can be different but complementary.

256 ***Fumonisins***

257 TMR samples that showed detectable levels of FBs usually contained compound feed, maize silage,
258 straw, grass silage, bagasse, defatted soya flour, dehydrated ryegrass and wheat silage more frequently
259 than the total set of samples, which includes positive and negative samples for FBs (Table S1).

260 Considering only the 60 FB-positive samples (with TMR composition available), higher concentrations of
261 FBs were found in samples that contained wheat silage (mean 775 µg/kg), sorghum silage (mean 758
262 µg/kg), barley (mean 733 µg/kg), and also dehydrated ryegrass, vetch silage and vetch hay (Table S2).
263 Accordingly, the concentration of FBs in the positive samples may be linked to the use of different kinds

of silages, plus barley, mainly, although barley was usually used in higher amount in samples also containing wheat and sorghum silage.

Moreover, the correlation analysis between FBs concentrations and the amount of TMR components showed a significant positive correlation between the level of FBs and the presence of wheat silage (r=0.31), barley (r=0.24), straw (r=0.19) and maize (r=0.16) in the TMR samples. However, PLS regression also pointed to the presence of barley, straw and maize and to immature corn silage as well. Both correlation analysis and PLS are somewhat dominated by the samples with higher FB concentrations. Compound feed was present in the four samples with higher concentrations. Wheat silage was rarely used by the farmers, but when used, the resulting samples contained FBs. In conclusion, as the components indicated by the correlations are different from those linked to the presence/absence of FBs, we can conclude that compound feed and silages are responsible for the presence of FBs in most samples, while, in particular, cereal silages may be related to the highest levels observed.

Deoxynivalenol

Samples that presented detectable levels of DON usually contained maize silage, straw, ryegrass silage, alfalfa silage, oat silage, rye silage and rapeseed as ingredients more frequently than the total set of samples (Table S1).

Considering only the samples that contained DON (29) (with a known TMR composition), we noticed that higher concentrations of toxin were determined in samples that contained cottonseed (mean of 418 µg/kg), immature corn silage (mean of 406 µg/kg), dehydrated alfalfa (mean 363 µg/kg), and also rapeseed, barley and grass silage (Table S2). Thus, DON contamination both in terms of incidence and concentration level seems to be linked to the use of different kinds of silages and cottonseed. However, cottonseed inclusion was correlated with the presence of immature corn silage, barley, alfalfa hay and dehydrated alfalfa. Thus, it is not possible to determine which of them contributed to the contamination. Similarly, the analysis of the correlation between DON concentration and levels of the different components in the TMR showed a significant positive correlation between the level of DON and the level of dehydrated alfalfa (r=0.40), immature corn silage (r=0.40) and cottonseed (r=0.35) and in a lower level straw, compound feed and rapeseed. As far as the PLS regression was concerned, the components correlated to DON were compound feed, cottonseed, maize and rapeseed, apart from immature corn silage. The three samples with higher concentrations contained dehydrated alfalfa and compound feed in

293 concentrations higher than average, which is relevant data. Cottonseed and immature corn silage were
294 rarely used by farmers, but they were present in one of the most contaminated samples. However, as the
295 components related to the level of contamination are different from the ones linked to the
296 presence/absence of DON, we can conclude that although maize, ryegrass or alfalfa silages could affect
297 the presence of DON in most samples, other components like immature corn silage, compound feed,
298 rapeseed, cottonseed or straw may be linked to the higher contamination.

299 ***Zearalenone***

300 Samples that contained detectable levels of ZEN had in common that they usually contained compound
301 feed, maize, grass silage, ryegrass silage, defatted soya flour and oat silage more frequently than the total
302 set of samples (Table S1).

303 Considering only the 25 ZEN-positive samples (with TMR composition available), we observed that
304 higher concentrations of ZEN were determined in samples that contained molasses (mean 269 µg/kg),
305 cottonseed (mean 216 µg/kg), sugar beet pulp (mean 200 µg/kg), oat silage (mean 185 µg/kg), alfalfa hay
306 (mean 128 µg/kg) and maize (mean 122 µg/kg), and also compound feed, maize silage and straw (Table
307 S2). Therefore, contamination both in terms of incidence and concentration level seems to be linked to
308 compound feed, maize, defatted soya flour and different kinds of silages.

309 Analysis of the correlation between ZEN concentration and levels of the different components in the
310 TMR showed a significant positive correlation between the level of ZEN and the levels of oat silage
311 (r=0.45), molasses (r=0.31), maize silage (r=0.25) and straw (r=0.18). Additionally, PLS regression
312 pointed to dehydrated alfalfa, straw, oat silage, maize silage and sugar beet pulp. The two samples with
313 higher concentration contained cereal silage plus compound feed or maize as main dry ingredients.
314 However, the components related to the highest levels of toxin are slightly different from the ones linked
315 to the presence/absence of ZEN because grass and ryegrass silages and defatted soya flour were not
316 present in highly contaminated samples.

317 Examining the results, it was difficult to reach definite conclusions about the influence of the ingredients
318 of TMR on the level of contamination. Other authors such as Cogan et al. (2017) stated than in some
319 samples, non-forage feeds appeared to contribute to the mycotoxin contamination, whereas in other
320 samples, the contributor was maize silage. For Romera et al. (2018), wheat bran, maize or barley seed
321 seemed to be related to the presence of FBs, DON and ZEN in cattle feed samples.

In conclusion, a reliable analytical method for the detection of multiple mycotoxins in a complex matrix was successfully developed and validated. This method was applied for the detection of *Fusarium* mycotoxins and their metabolites in TMR samples for two years. A total of 58% of the samples presented at least one mycotoxin, with FBs being the most frequently found. Moreover, in 20% of the samples, more than one mycotoxin was detected. The level of contamination was always below the guidance levels established by the EU. However, the presence of mycotoxins in animal feed and in feed ingredients should be regularly monitored to prevent health hazards in animals and humans. In addition, special attention must be paid to the co-occurrence of mycotoxins due to their possible synergistic and additive effects. To our knowledge, few studies have been carried out analysing TMR. Thus, this study provided useful information about the level of mycotoxin contamination in final products, as they are consumed by animals. Although it is difficult to draw definite conclusions, the use of some silages and certain concentrates in the formulation of TMR is probably related to the presence of *Fusarium* mycotoxins.

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Conflict of interest

The authors declare no conflict of interest.

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477 **Table 1.** HPLC-MS/MS conditions.

Analyte	Retention time (min)	Ionization mode	Precursor ion *m/z* (fragmentor voltage (V))	Product ion *m/z* (collision energy (V))
DON-3-Glc	2.7	ESI^-	476.2 (85)	**297.1 (5)**[a]
				248.9 (25)
				203.1 (25)
DON	2.8	ESI^+	297.2 (65)	231.1 (15)
				203.1 (15)
				174.9 (25)
$^{13}C_{15}$-DON	2.9	ESI^+	312.2 (125)	**263.1 (5)**
				245.0 (5)
				216.2 (15)
				186.0 (15)
15-ADON	3.7	ESI^+	339.2 (125)	**321.1 (5)**
				261.1 (5)
				137.0 (5)
3-ADON	3.7	ESI^+	339.2 (105)	**231.1 (5)**
				203.0 (15)
				175.1 (25)
FB_1	4.2	ESI^+	722.4 (175)	352.1 (30)
				334.1 (40)
FB_2	4.7	ESI^+	706.5 (125)	**336.4 (35)**
				318.4 (45)
β-ZEN	4.8	ESI^-	323.2 (125)	174.0 (40)
				160.0 (40)
				129.9 (40)
α-ZEN	5.0	ESI^-	323.2 (125)	174.0 (40)
				160.0 (40)
				129.9 (40)
ZEN	5.0	ESI^+	319.2 (85)	**301.1** (5)
				283.1 (15)
				157.0 (35)
$^{13}C_{18}$- ZEN	5.0	ESI^+	337.3 (65)	**319.2 (5)**
				301.1 (5)
				215.0 (25)
				199.0 (15)

478 [a]Transitions in bold were used for quantification. The rest were used for confirmation. Both must be

479 monitored simultaneously in the sample to guarantee the presence of an analyte in the sample.

480

481 **Table 2.** Method parameters for DON, DON-3-Glc, 3-ADON, 15-ADON, ZEN, α-ZEN, β-ZEN, FB_1 and
482 FB_2 determination in total mixed ration using acetonitrile (79%)/water (19%)/acetic acid (1%) as
483 extraction solution and quantification with HPLC-MS/MS.

Mycotoxin	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	N	Spike level (µg/kg)	Recovery[c] (%)	RSDr[d] (%)
DON	0.75	1.0	3	500	103.6±10.3	9.9
			5	2000	129.4±34.8	26.9
			3	5000	103.4±8.3	8.0
DON-3-Glc	0.75	1.0	3	100	92.7±8.0	8.7
			5	400	112.4±28.6	25.5
			3	1000	110.8±5.6	5.1
3-ADON	0.3	0.5	3	100	68.4±6.6	9.7
			5	400	98.8±6.6	6.7
			3	1000	96.5±2.2	2.3
15-ADON	0.3	0.5	3	100	102.6±8.7	8.5
			5	400	82.7±15.8	19.1
			3	1000	74.2±3.7	5.0
ZEN	0.25	0.5	3	20	104.3±6.3	6.0
			5	100	111.6±12.6	11.3
			3	500	80.8±6.5	8.0
α-ZEN	0.25	0.5	3	50	91.7±12.7	13.8
			5	100	96.7±11.7	12.1
			3	250	87.6±4.1	4.6
β-ZEN	0.25	0.5	3	50	72.0±7.9	10.9
			5	100	96.7±11.7	12.1
			3	250	87.6±4.0	4.6
FB_1	30	45	3	1000	72.0±3.4	4.7
			5	2000	76.4±3.4	4.5
			3	5000	97.5±7.6	7.8
FB_2	30	45	3	500	96.2±4.7	4.9
			5	1000	110.7±6.9	6.3
			3	2500	119.1±7.0	5.9

484 [a]LOD = limit of detection
485 [b]LOQ = limit of quantification
486 [c]Mean value ± standard deviation
487 [d]RSDr = relative standard deviation

488 **Table 3.** *Fusarium* mycotoxins detected in the TMR samples analysed (n=193).

Analyte	GV (µg/kg)	No. of positive samples (%)	Mean (µg/kg)	Range (µg/kg)
DON	5000	32 (16.6%)	260	40-790
15-ADON		18 (9.3%)	28	9-50
DON-3-Glc		3 (1.6%)	46	10-72
ZEN	500	31 (16.0%)	90	3-430
FB_1+FB_2	50000	66 (34.2%)	600	350-1900

489 GV = guidance value for feedstuffs established in the European Commission Recommendation
490 2006/576/EC.

Table 4. Number of positive samples, mean values and median of DON, ZEN and FBs concentrations detected in the samples from each sampling area.

Area	No. of total samples	DON			ZEN			FBs		
		No. of positive samples (%)	Mean (µg/kg)	Median (µg/kg)	No. of positive samples (%)	Mean (µg/kg)	Median (µg/kg)	No. of positive samples (%)	Mean (µg/kg)	Median (µg/kg)
01	48	6 (12.5%)	180	60	7 (14.6%)	80	20	12 (25.0%)	480	470
03	47	6 (12.8%)	360	330	3 (6.4%)	80	10	1 (2.1%)	420	420
04	43	7 (16.3%)	350	300	9 (20.9%)	20	7	23 (53.5%)	590	470
08	12	3 (25.0%)	270	320	0	-	-	4 (33.3%)	440	470
25	43	10 (23.3%)	180	170	12 (27.9%)	150	110	26 (60.5%)	690	500

* Castilla-León: area 01 and area 03; Cantabria: area 04; Galicia: area 08; Cataluña: area 25